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STUDY OF THE EFFECT OF AMNIOTIC MEMBRANE GRAFTING ON CORNEAL ULCERS

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CERTIFICATE

This is to certify that this dissertation entitled **"STUDY OF THE EFFECT OF AMNIOTIC MEMBRANE GRAFTING ON CORNEAL ULCERS"** is a bonafide record of the research work done by **Dr. GAYATRI MURUGAN** Post graduate in Regional Institute of Ophthalmology, Madras Medical College and Research Institute, Government General Hospital,Chennai-03, in partial fulfillment of the regulations laid down by The Tamil Nadu Dr.M.G.R. Medical University for the award of M.S. Ophthalmology Branch III, under my guidance and supervision during the academic years 2009-2012.

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ABBREVIATIONS

AMG - AMNIOTIC MEMBRANE GRAFT

AM - AMNIOTIC MEMBRANE

TKP - THERAPEUTIC KERATOPLASTY

CONTENTS

PART – 1

INTRODUCTION

The first reported use of fetal membranes in skin transplantation was by Davis in 1910. In 1913, Sabella used amniotic membrane on burned and ulcerated skin surfaces and observed lack of infection, marked decrease in pain, and increased rate of re-epithelialization of traumatized skin surface. Others have demonstrated the use of amniotic membrane as a biological dressing for open wounds including burns and chronic ulceration of the legs.[1]

The first use of amniotic membrane transplantation (AMT) in ophthalmology was by De Roth in 1940 who reported partial success in the treatment of conjunctival epithelial defects after symblepharon. Sorsby and Symons in 1946 found that patients with caustic burns of the conjunctiva with corneal involvement could be treated successfully using amniotic membrane. Little else regarding AMT appeared in the ophthalmic literature until 1995 when Kim and Tseng used AMT for ocular surface reconstruction of severely damaged corneas in a rabbit model. Since that experimental study, AMT has been used for persistent corneal epithelial defects, corneal ulcers,leaking filtering blebs after glaucoma surgery, pterygium surgery, conjunctival surface

reconstruction, bullous keratopathy, chemical or thermal burns, ocular surface reconstruction with or without limbal stem cell grafting, and in patients with ocular cicatricial pemphigoid or Stevens-Johnson syndrome[5].

ANATOMY OF THE CORNEA

The cornea is a transparent, vascular watch glass like structure forming the anterior one sixth of the eyeball. It forms the principal refractive surface, contains the intraocular pressure and provides a protective interface with the environment. These functions can be sub-served by virtue of a sub-structural organization. Histologically, the cornea is composed of five layers (Fig.1).

Epithelium

Bowman's membrane

Stroma or substantia propria

Descemet's membrane

Endothelium

CORNEAL EPITHELIUM:

It is stratified squamous and nonkeratinized.It is 50-90 micron thick and consists of 5 or 6 layers of nucleated cells.The epithelial cells are arranged in three layers.

DEEP ZONE:

It consists of single layer of basal columnar cells and forms the germinative zone.

MIDDLE ZONE:

It comprises of 2 to 3 layers of polyhedral cells called wing cells which are convex anteriorly and cap the basal cells.

SUPERFICIAL ZONE:

It has 2 to 3 layers of flattened nucleated cells called squamous cells.

The integrity of the epithelium plays an important role in protecting the cornea against most of the pathogens responsible for corneal ulcers. Any disruption in the epithelium predisposes the cornea to infection.

BOWMAN'S LAYER:

It is a narrow, acellular, homogenous zone, 8 to 14 micron thick, immediately subjacent to the basal lamina of the epithelium. It is relatively resistant to trauma due to the compact arrangement of collagen but once destroyed, it can't be regenerated.

STROMA OR LAMINA PROPRIA:

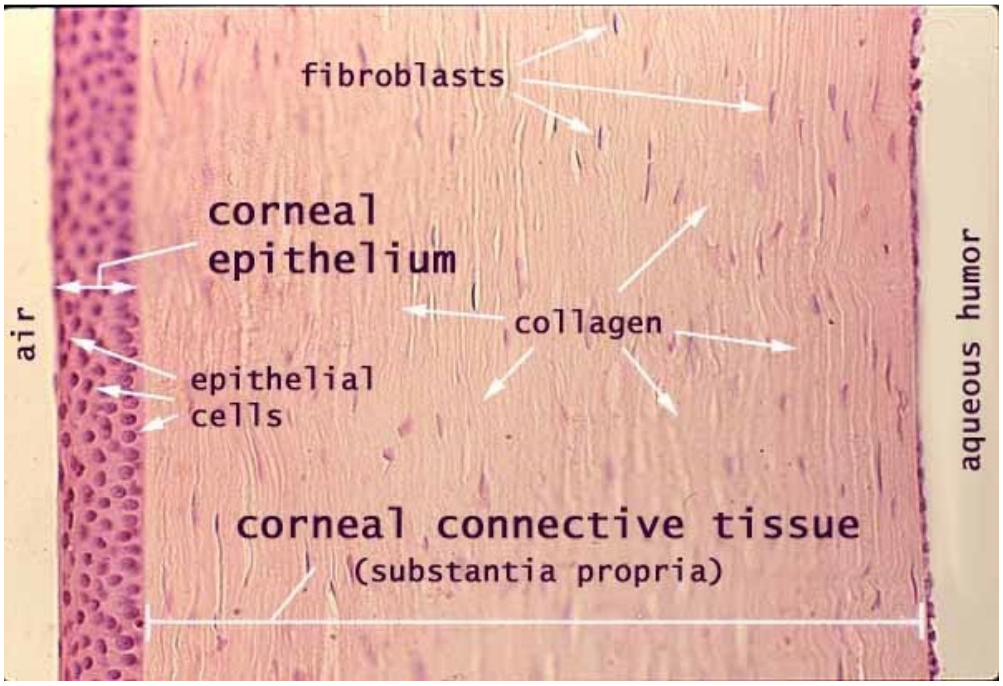
It is around 560 microns thick (90% of the thickness of the cornea) and comprises of regularly arranged lamellae of collagen bundles in a proteoglycan ground substance with cells called keratocytes. Collagen makes up 71% of the dry weight of the cornea. One of the unique features is the regular alignment of collagen fibrils. Another unique feature is the consistent diameter of the collagen fibrils (27-35nm).The relatively small diameter of the corneal collagen fibres is the result of elevated levels of type 5 collagen. It makes up 15-20% of the total. The size also depends on the association of the fibrils with proteoglycans. Decorin, lumican and fibromodullin are all known to associate with collagen fibrils and limit the size of the fibril. The lamellar arrangement is less precise in the anterior stroma.

DESCEMET'S MEMBRANE

It is a 10-12 micron thick basal lamina produced by the endothelium. It's peripheral termination is marked by the Schwalbe's line. The major protein is type four collagen. Descemet's membrane readily regenerates following injury.

ENDOTHELIUM:

The endothelium is a single layer of cells located in the posterior part of the cornea. It permits the passage of nutrients from the aqueous humor into the cornea. The endothelium is the major cell layer responsible for maintaining the relatively low level of stromal hydration necessary for corneal transparency.[7]



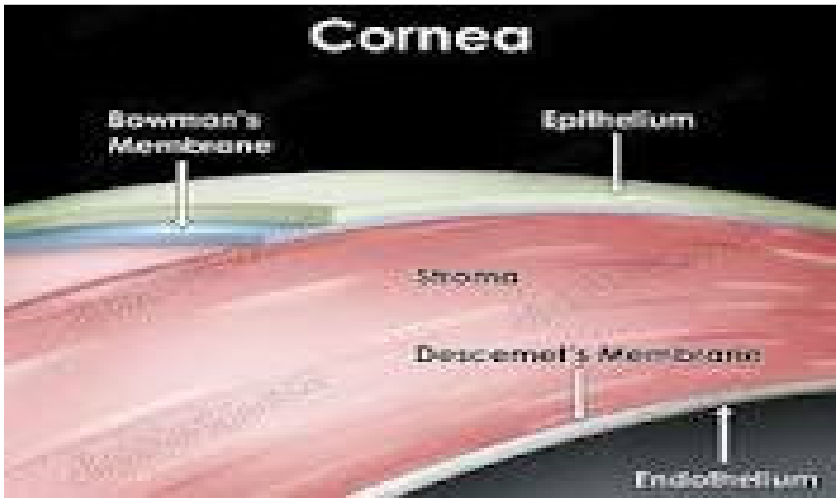


Fig.1 (a & b) - Anatomy of the cornea

PATHOLOGY OF CORNEAL WOUND HEALING

The primary function of the corneal epithelium is to form a barrier to invasion of the eye by pathogens and for uptake of excess fluid from the stroma. Injury to the cornea may be accidental or iatrogenic in origin. After abrasion the first 4-6 hours after epithelial injury is the initial latent phase where no appreciable decrease in size of the wound occurs. The basal and squamous cells in the vicinity of the wound show thickening and separation. Neutrophils accumulate along the wound edge 3 hours after the injury as does the thinning of the epithelium to a single layer. The leading edge of the migrating cells is only one cell thick. The cells enlarge and the epithelial sheets begin to migrate by amoeboid movements across the defect until it is completely covered. A circumferential migration of 3-6 leading fronts progressed towards the center. They meet and merge and form a multilayered sheet. A wound of around 6 mm in diameter closes within 48 hours and the rate of migration is 60-80 micro meters per hour.

The basal cells play a key role, the source of which is the limbal basal stem cells. The centripetal migration was due to the inward drawing of cells by preferential desquamation of central corneal epithelial cells. It is interesting to note that the healing rates for larger

defects were more rapid than for smaller defects. This is attributed to greater proliferative response of cells in the peripheral cornea and limbus than the central cornea. An important aspect of wound healing is the reformation of adhesion complexes to the underlying connective tissue. Initial linkage is between cytoplasmic actin filaments of the migrating epithelial cells to the extracellular matrix proteins like fibronectin, fibrinogen-fibrin, laminin, tenascin, and integrin. Reformation of adhesion complexes gradually occurs from the periphery to the center.

Stromal wound healing:

After injury,keratocytes are capable of phagocytosis of collagen fibrils and synthesis and secretion of collagen, glycosaminoglycan ground substance , collagenase and collagenase inhibitors. Within hours a cascade of response to cytokine occur, polymorphonuclear cells occur around areas of cellular necrosis. Immediately following injury initial keratocyte apoptosis occurs, within 12 hours proliferation and migration of residual activated keratocyte occur that repopulate the depleted stroma. TGF β reduces corneal fibrosis. The healing process is initiated by multiple cytokines and growth factors like IL-1, TNFα, Bone morphogenic proteins 2 and 4, Epidermal growth factor(EGF), Platelet derived growth factor(PDGF) from the corneal epithelium and basement

membrane. There is also removal of damaged tissue by the plasminogen-activator/plasmin system, metalloproteinases (MMPs). These MMPs from polymorphonuclear sites are involved in epithelial migration, initial stromal degradation and removal of damaged tissue. MMP2 is involved in the prolonged process of collagen remodeling in the stromal repair tissue. MMP9 in the epithelium is believed to be involved in the degradation of basement membrane that precedes corneal ulceration, as well as controlling the resynthesis of basement membrane.

Return of normal structure and function may take months or even years, depending upon the nature of injury or surgery.[1,2]

Factors protecting the cornea:

An intact corneal epithelial surface with its tight junctions formed by desmosomes and hemi desmosomes protects the surface of eye from infectitious agents. The eyelid provides a physical barrier to the organism. The tear film contains antimicrobial enzymes, immunoglobulins, lysozyme, lactoferrin. The normal ocular floraprovides a balance to prevent overgrowth of exogenous organisms. The conjunctiva contains subepithelial mucosal associated lymphoid tissue.

Healing Response To Corneal Infection

In keratitis, entry of organisms result in diffusion of toxins and enzymes. Polymorphonuclear leukocytes arrive at the corneal wound site. Stromal damage from bacterial and neutrophil enzymes facilitate bacterial invasion of cornea. There is a progressive tissue necrosis and sloughing of epithelium and stroma which varies with the virulence of the organism and toxin. The necrotic base of the ulcer is surrounded by heaped up tissue. The host cellular and humoral defence mechanisms retard bacterial replication, promote phagocytosis and halt destruction of stromal collagen.

In the healing phase, the epithelium resurfaces the central area of ulceration and the necrotic stroma is replaced by scar tissue produced by fibroblast. These fibroblasts are derived from keratocytes. New epithelium slowly resurfaces irregular bases. Bowmens layer does not regenerate bur replaced with fibrous tissue. New blood vessel are directed towards area of ulceration to deliver humoral and cellular components to promote healing. These gradually disappear and may leave ghost vessels. The fibrous scar tissue results in corneal opacity which may gradually fade over time.[7,25]

EPIDEMIOLOGY OF BACTERIAL AND FUNGAL CORNEAL ULCERS

FUNGAL:

In the Northern part of India, Nepal and Coastal Karnataka Aspergillus species are found to be frequently involved whereas in Southern part of India, Fusarium species is reported as the leading cause of keratitis.

In general ,Aspregillus species are common in India as shown by many studies. Candida is very rare in India as causative organism except in eyes predisposed to it.

Fungi common in cooler parts of the world is Candida whereas Aspergillus and Fusarium are common in warmer climates. Fungi rarely infect intact cornea. They are opportunistic pathogens. They infect when host immunity is deranged either locally or when systemic immune deficiency exists.

Fungi are ubiquitous, their light spores produced in huge numbers are disseminated and have remarkable ability to germinate and grow on almost any organic substance.

Fusarium species are primarily plant pathogens and keratomycosis caused by them are common in agricultural workers.

BACTERIAL:

There are four principal groups of bacteria involved; Micrococcus (staphylococcus, micrococcus), the streptococcus species, the pseudomonas species and the enterobactericiae (proteus and klebsiella). Ambient temperature and humidity determine the organism in the environment[3].

Streptococcus species is the commonest isolate in South India. Staphylococcus is among the frequent organism in the United States. Pneumococcus is associated with chronic dacryocystitis. Pseudomonas occurs in soft contact lens users. Moraxella in malnourished individuals. Neiserria can cause keratitis secondary to conjunctivitis. Perforation is common. A typical mycobateria can cause keratitis following Lasik.

Neiserria gonorrhea, Corynebacterium and H.Influenza can penetrate intact epithelium.

PATHOGENESIS OF CORNEAL ULCERS

The epithelium plays an important part in protecting the cornea against most pathogens.Any breach in the epithelium will predispose the cornea to infection. The pathogenic mechanisms of fungi include,

1. Direct physical damage caused by invasion and growth of fungal elements.

2. Damage from infiltrating leucocytes.

3. Damage produced by fungal toxins and enzymes.

In corneal fungal infections, clinical manifestation may occur as quickly as 24-48 hours or may be delayed for 10-20 days, potentially allowing the extensive fungal replication before deduction by the host.

Infiltration of the host leucocytes particularly neutrophils is an important component of corneal damage produced in keratomycosis. Fungal hyphae are large enough to preclude ingestion by neutrophils, however neutrophils are known to destroy fungal hyphae and probably also damage surrounding tissue by frustrated phagocytosis with consequent extracellular release of lysosomal enzymes and oxygen metabolites.

Though a breach in the epithelium is essential for penetration, there are certain bacteria that can penetrate intact epithelium like N.Gonorrhoea, N.menigitides, C.diphtheriae and H.influenza.

In the pathogenesis of bacterial ulcers there are various factors which play a role. Adhesins are microbial proteins that adhere to the host cell and promote entry into the cell, induce cytokine production and may act as toxins directly. Pseudomonas has many virulence factors; Pili, fimbriae and flagella for adherence to the host cell and also help in bacterial motility important for bacterial dissemination of infection and also products like elastase and protease. In addition Pseudomonas and N.Gonorrhoeae is guided by glycocalyx a biological slime that enables them to adhere to susceptible cells, producing slime aggregates that are resistant to phagocytosis. In addition lysosomal enzymes and oxidative substances produced by neutrophils, keratocytes and epithelial cells also contribute to the destruction. Lipopolysaccharide composing the exotoxin in the cell wall also play a role.

CLINICAL FEATURES

SYMPTOMS:

The rich innervation of the cornea makes pain the most common symptom. Movement of the eyelids over ulcerated corneal epithelium intensifies the pain.

There is a variable decrease in vision and also associated watering, photophobia and blepharospasm. Discharge is usually rare unless there is an associated conjunctivitis such as with gonococcus, pnemococcal and Haemophilus infections.

FUNGAL KERATITIS (Fig.2)

Keratitis caused by filamentous fungi:

1. More common in young individuals involved in outdoor activities
2. There is always a history of trauma with vegetable matter.
3. Incubation period is 24-48 hrs, it may involve any area of cornea
4. No evidence of prior ocular surface disorder like dry eyes, herpes or neuroparalytic keratitis is present.[(2)]

SIGNS:

1. Greyish white infiltrate with hyphate margins elevated above the surface of cornea, surrounded by satellite lesions.
2. Ulcer base may have a dry texture.
3. Ulcer margins are irregular and elevated; may demonstrate irregular fine,linear infiltration branching into the surrounding cornea.
4. Satellite lesions are discrete, stromal abscesses that surround the ulcer and are separated by clear cornea.
5. Clear endothelial plaques are seen composed of inflammatory cells.
6. Immune ring composed of antigen-antibody complex.
7. Hypopyon is commonly associated feature even with a small ulcer but is not specific.

Keratitis caused by non-inflamentous fungi:

This occurs commonly and exclusively in eyes with pre-existing corneal surface abnormalities like keratitis sicca, neuroparalytic keratitis, herpes affected eyes[8].

1. Ulcers usually occurs at the area of exposure; at the junction of superior 2/3 and inferior 1/3.

2. Keratitis is more localized ,may have an expanding, small discrete infiltrate.

3. No hyphate margins and edges not feathery.

4. Satellite lesions are not usually seen

BACTERIAL ULCERS (Fig.3)

1. An epithelial defect with underlying stromal infiltrate

2. An anterior chamber reaction and a hypopion which is characteristically sterile and mobile.

3. The base is wet

4. Gram positive cocci cause round ulcers with grayish white infiltrates with distinct borders and minimal surrounding edema.

5. S.pneumoniae presents with a deep, oval central ulcer with serpiginous edges, a classic serpiginous corneal ulcer.

6. Severe conjunctival hyperemia and chemosis in Nieserria.

7. Nocardia shows crystalline bodies

8. Dark hypopion with elevated intraocular pressure in Listeria[7].

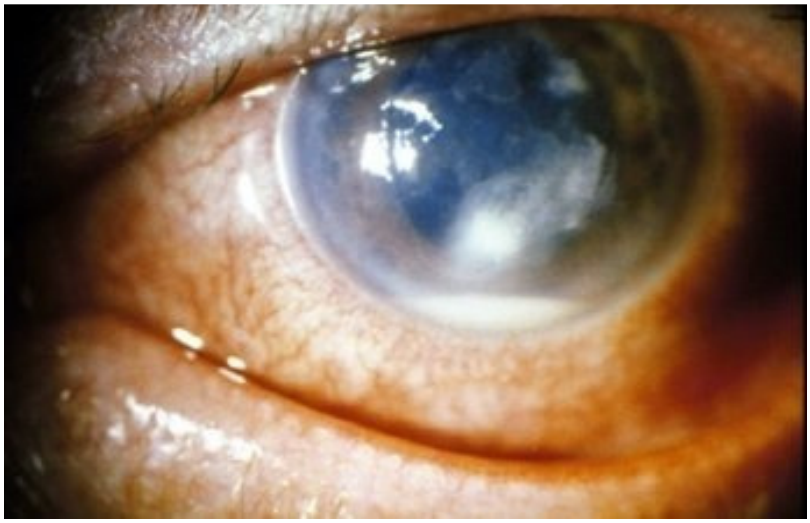


Fig.2 - Fungal corneal ulcer

Fig.3 - Bacterial ulcer

Table – 1 : Classification of ulcer severity

FEATURE	MILD	MODERATE	SEVERE
Size Of Ulcer (mm)	<2	2-5	>5
Depth Of Ulcer (%)	<20	20-50	>50
Infiltrate	Superficial	Extension Upto Mid Stroma	Deeper Than Mid Stroma
Scleral Involvement	Not Involved	Not Involved	May Be Involved

TREATMENT OF CORNEAL ULCERS

MEDICAL

ANTIFUNGALS

Amphotericin B:

It is a polyene antibiotic derived from strains Streptomyces nodosus.

Mechanism of action

The antibiotic binds to ergosterol present in the cell membranes, increase the permeability of cytoplasmic membranes, thereby permitting leakage of essential intracellular constituents. It is fungicidal at high concentration and fungistatic at low concentration.

Natamycin:

This is a small polyene antibiotic first isolated in 1958.

Mechanism of action

It binds to ergosterol present in the cell membranes, increase the permeability of cytoplasmic membranes, thereby permitting leakage of essential intracellular constituents.

Voriconazole:

It inhibits fungal cytochrome-P450 mediated 14α lanosterol demethylation mediated ergosterol synthesis present in the fungal cell wall. Fluconazole is another azole derivative used.

Chlorhexidine:

Mechanism of action

It acts by attacking and rupturing the cell membranes. It inhibits both cation transport and membrane bound ATP in the cell membrane.

ANTIBACTERIALS

Flouroquinolones:

They inhibit the action of bacterial DNA gyrase an enzyme essential for bacterial DNA synthesis. The commercially available flouroquinolones are highly ffective against pseudomonas aeruginosa and strains resistant to other agents.

Aminoglycosides:

They have a selective affinity for the bacterial 30s and 50s ribosomal subunits to produce a non-functional 70s initiation complex

that inturn results in the inhibition of bacterial cell protein synthesis. Commonly amikacin and gentamycin are used. They are effective against aerobic and faucultative gram negative bacilli.

Cephalosporins:

They inhibit the third and final stage of bacterial cell wall synthesis by preferentially binding to one or more penicillin binding proteins that are in the cytoplasmic membrane. Cephazolin and ceftazidime is commonly used. Fortified preparations are generally used. Intrastomal and intracameral antibiotics are also being tried.

SURGICAL THERAPY:

In case there is a perforation,

- Cyanoacrylate glue may be tried for perforations less than 3mm
- For larger perforations a patch graft may be done.
- Penetrating keratoplasty is indicated in either medical treatment failures, in case of impending perforations and frank perforations more than 2mm.

In this procedure the entire thickness of the cornea is removed and replaced with an appropriate size corneal button from the donor. The procedure by itself has many complications.

Conjunctival flaps

It is generally indicated when medical therapy and surgery like amniotic membrane transplantation, patch grafting or penetrating keratoplasty fail. It may be partial or complete. The flap should cover the diseased area tension on the flap should be avoided to prevent retraction. It should not contain Tenon's membrane. Any hole in the flap compromises the success of the procedure. The cornea under the flap should be mechanically debrided. The flap should be atleast one mm larger than the lesion on the cornea. One should not compromise the integrity of the flap be needling into the area of the intended flap. A combination of gentle blunt and sharp dissection is used with particular attention to avoid grasping the conjunctiva. A partial flap may be Van Lint hood peripheral flap, Racquet and bucket handle flap. Total conjuntival flap or Gunderson's flap. A peritomy is done, the upper forniceal conjunctiva has a larger area as the upper the fornix is deep, the distance from the limbus to limit of the upper fornix is usually 16-18mm.The flap is sutured to the sclera. The area above the upper

limbus can be left uncovered, its epithelializaion is usually very rapid and complicated.

The results are usually very good leading to stabilization of the cornea, a reduction of inflammation and pain. The conjunctiva transparent within 8 weeks.

Complications include button holes in the flap, necrosis of the flap and corneal melting and necrosis under the flap.

Amniotic membrane grafts are becoming very popular now.

AMNIOTIC MEMBRANE GRAFTING

Amniotic membrane transplantation is indicated for numerous ocular surface disorders, the spectrum of which has been expanding recently. The first report on the use of amniotic membrane for ocular disorders was by DeRoth in 1940.

ANATOMY OF THE AMNIOTIC MEMBRANE:

It has five layers,

Epithelium

Basement membrane

Avascular connective tissue

Compact layer

Fibroblast layer

Spongy layer

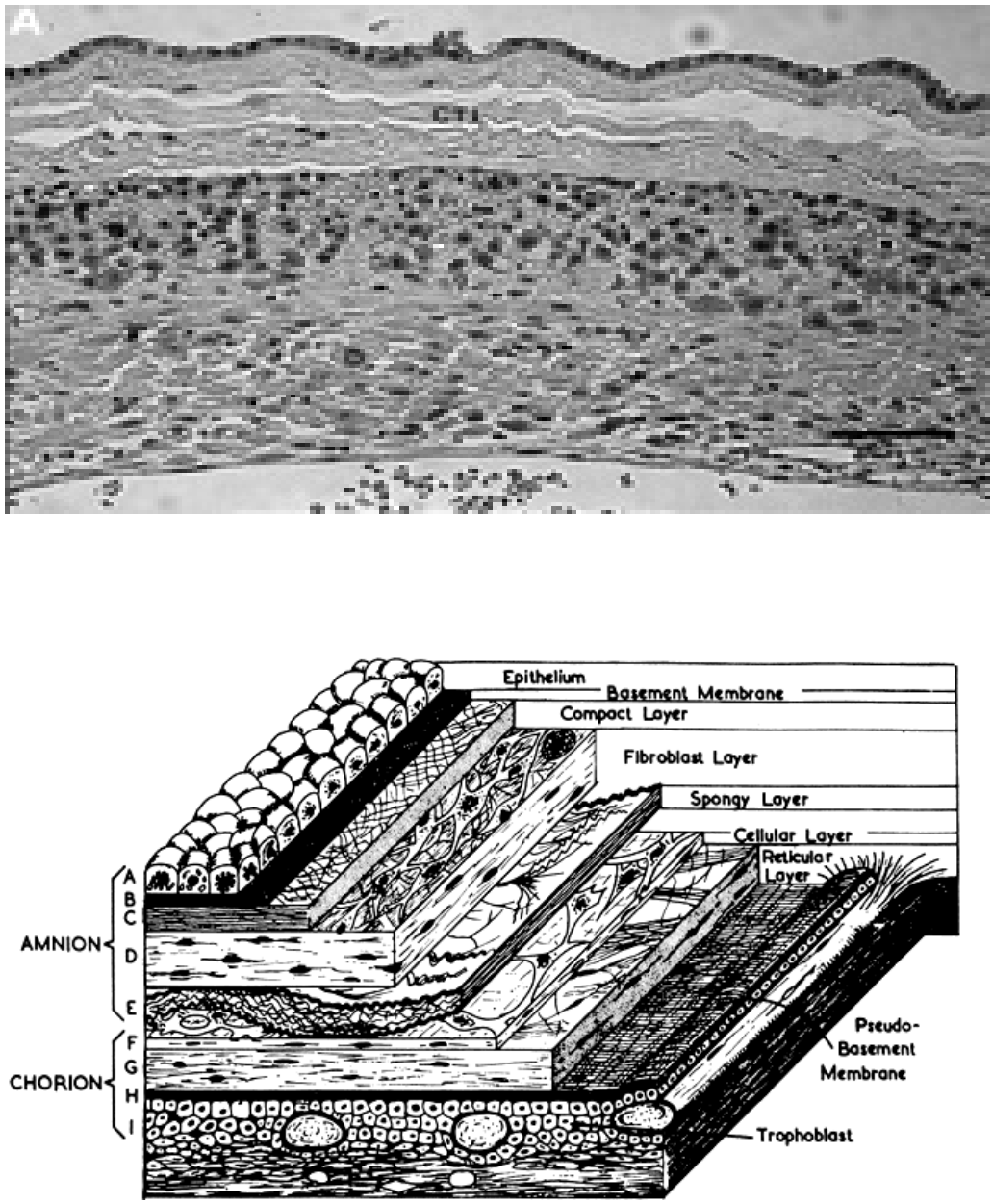


Fig4 Anatomy of the Amniotic membrane

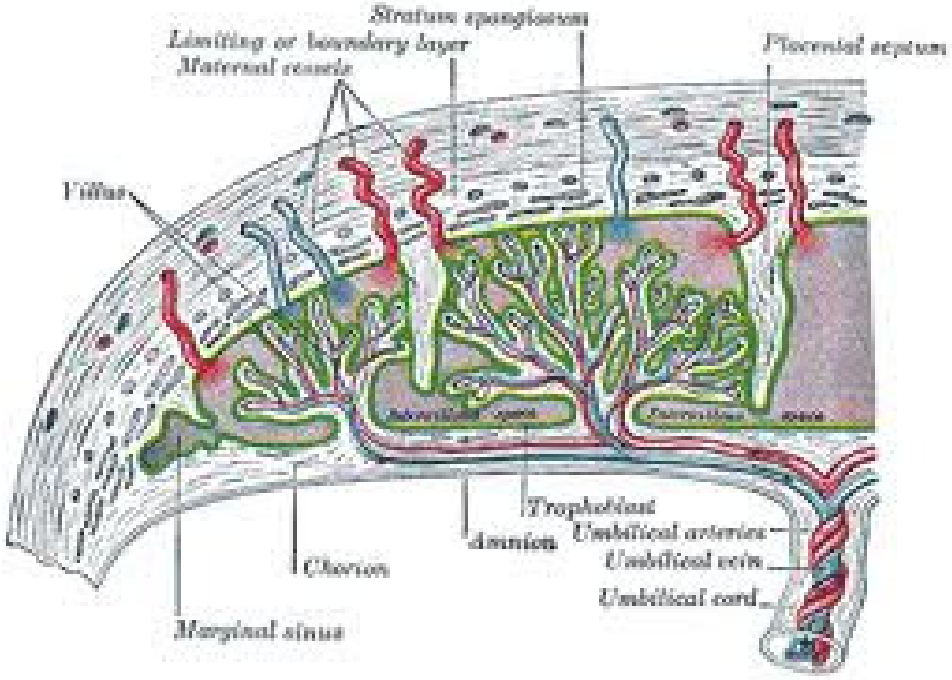


Fig 5 Amnion as part of the placenta

The thickness of the membrane is 0.02-0.5mm.The compact layer is the strongest. The fibroblast layer is the thickest.(fig4)

FUNCTION:

There is growing evidence that biologic factors including collagen and cytokines may make a major difference between a soft contact lens and amniotic membrane. The amniotic membrane is believed to provide a superior substrate for migration of epithelial cells, reinforce adheshion of basal epithelial cells, promote epithelial differentiation, prevents apoptosis of epithelial cells and reduces neovascularization and fibrosis by reduction of inflammatory cell infiltration.

Both collagen four and seven, components of corneal and conjunctival epithelial basement membrane are present in the basement membrane of amniotic membrane. In addition, amniotic membrane contains collagen three, one, two, five, fibronectin and laminin[15]. Cytokines promote epithelialization and reduce inflammation, scarring and neovascularization. They are found in both the epithelium and stroma but believed to be synthesized predominantly by the epithelium. They are also present in cryopreserved amniotic membrane and

approximately 50% of the epithelial cells can be viable for several months. Hence it is possible that the amnion epithelial cells continue to produce cytokines after transplantation. Nerve growth factor, epidermal growth factor, keratocyte growth factor and hepatocyte growth factor may play a role in promoting epithelialization. The mechanism by which it reduces scarring, inflammation and angiogenesis are more complex. It uniquely suppresses transforming growth factor β signaling resulting in reduction in fibroblasts and scarring. It also traps inflammatory cells of the monocyte macrophage system. Fresh human amniotic cells produce antiangiogenic agents, interleukin 1 receptor antagonist, all four matrix metalloproteinase inhibitors, interleukin ten and thrombospondin[24].

IMMUNE PREVILIGE:

Unlike other allograft transplantation it does not require the administration of systemic immunosuppressive therapy to prevent immune rejection. The amniotic membrane typically dissolves in 3-5 weeks. It is believed that the amniotic membrane is an immune previliged tissue. The epithelium, stroma and fibroblasts of cryopreserved amniotic membrane contains HLA class 1 and 2 antigens, but the amnion has the ability to suppress alloreactive T cells. Furthermore amnion expresses HLA-G, a nonclassic major

histocompatibility complex class one molecule expressed in the extravillous cytotrophoblast at the feto-maternal interface, that protects the fetus from maternal cellular immunity. This antigen along with other immunoregulatory molecules plays a role in suppressing the infiltration of CD4+ and CD8+ T cells into amnion.

PREPARATION AND STORAGE:

Both in vivo and in vitro studies suggest there is very little difference between fresh and cryopreserved amniotic membrane. The amniotic membrane shows little morphological change after cryopreservation in 50% glycerol. The cryopreserved membrane is typically stored at -80 degree Celsius on a nitrocellulose paper in 50% glycerol with epithelial side up(fig.6).The epithelial side is smooth and can be distinguished from the sticky stromal side by touching it with a Weck-cel sponge. It can be stained with Lissamine green for better visualization of edges and folds during the procedure. The colour returns to normal in 120 minutes.

It is obtained from the placentas of donors undergoing elective caesarian section. Donor screening to exclude the presence of HIV 1 and 2, hepatitis B, hepatitis C, human T lymphocyte virus 1 and 2 and syphilis in the donor serum are performed before harvesting the placenta and repeated six months later to guard against transmission of blood borne pathogens. The placenta is transported under sterile conditions to a lamellar –flow hood and cleaned of blood clots with sterile Earle's balanced saline solution containing 50µg/ml of penicillin, 50µ/ml of streptomycin,100µ/ml of neomycin and 2.5µg/ml of amphotericin (fig.6).The amnion is separated from the chorion by blunt dissection and placed on a nitrocellulose paper with the epithelium side up. The nitrocellulose paper is then cut in pieces 4 × 4 centimeters and stored at -80 degree Celsius in a sterile vial containing Dulbecco's modified Eagle medium and glycerol in the ratio 1:1.If for less than four weeks it can be stored at -20 degree Celsius. It is thawed before use. There is also a heat dehydrated one which can be stored at room temperature and rehydrated with saline minutes before use. It does not need a carrier sheet. Both fresh and preserved AM have been found to function equally well when transplanted onto the ocular surface. However, there are certain concerns when using fresh AM. Ideally, serologic tests on the maternal donor must be done both at the time of procurement of the

donor tissue and again six months later. This dual testing eliminates the slightest risk of disease transmission. With the fresh AM the time interval from tissue procurement to transplantation is short and prevents repeat testing of the donor. Patients have to be brought to the hospital at a short notice unlike with preserved AM, which allows more flexibility in scheduling surgery. A distinct disadvantage is wastage of unused tissue with non-preserved AM as opposed to frozen AM where up to 30 grafts can be prepared from one placenta[26].

The epithelial cells in fresh or preserved AMG are nonviable. The viability of amniotic epithelial cells may be associated with low-grade inflammatory response. The drawback with the preserved AM is the need for a -70 ^{o} refrigerator, which precludes its use outside big institutions.[7]

Fig.6 - Processing and storage of AMG

SURGICAL TECHNIQUE:

There are several basic techniques described for amniotic membrane transplantation. The clinical indication will indicate the most appropriate technique.

- Graft or inlay technique
- Patch or overlay technique
- Grafts (one or more layers) as a stromal filling technique
- Ex vivo limbal stem cell transplantation

Cryopreserved amniotic membrane is usually stored on nitrocellulose paper with the stromal (sticky) side down. If there is confusion, a blunt forceps can be used to draw up a vitreous like strand.

GRAFT OR INLAY TECHNIQUE:

This involves placing a membrane with epithelial side up. One or multiple layers are placed depending upon the depth of the defect. The membrane is sutured either to the cornea with 10-0 and bandage contact lens then placed. In this technique there is less corneal transparency. The inlay technique involves placement of the amniotic membrane graft into

the corneal ulcer secured into place by interrupted sutures without extending beyond the edge of the epithelial defect. The amniotic membrane thus acts as a basement membrane to which epithelialization may take place over it from the surrounding epithelium. Therefore, persistent corneal defects or ulceration may be treated prior to or after perforation. Mejia, et al. successfully used larger AMT for treatment of symptomatic bullous keratopathy by leaving peripheral cornea uncovered to promote epithelialization over the graft. They suggested that AM used in this fashion may be an alternative to conjunctival flap in corneas with poor visual potential. In using this technique, however, the amniotic membrane graft becomes trapped under the healed corneal epithelium and may limit corneal transparency for several months or more affecting vision. (Fig.7)

PATCH OR OVERLAY TECHNIQUE:

The amniotic membrane is placed with epithelial side down. In the overlay technique, the entire corneal surface including the limbus is covered with the amniotic membrane graft. Here the amniotic membrane functions primarily as a biological contact lens. The graft protects regenerating epithelium from the frictional forces of the eyelid and palpebral conjunctiva while at the same time appears to allow

adequate oxygen permeability and moisture to the epithelium. Corneal transparency remains when the graft eventually detaches or dissolves. This method has been used successfully in cases of stem cell deficiency of various causes and in cases of persistent epithelial defect unresponsive to medical therapy and surgically induced epithelial defects. Gris, et al. propose that this method may be a safe alternative that some patients may prefer over tarsorrhaphy when medical treatment has failed. Both the inlay and overlay techniques may be used together[11]. (Fig.8)

LAYERED FILLING TECHNIQUE:

Several layers of amniotic membrane may be used to pack into a deep ulcer cavity. The orientation of the deeper layers appeared to be unimportant, the most superficial layer is placed with the basement membrane side up. Only the superficial layer needs to be sutured in place similar to the inlay technique[18].

EX VIVO LIMBAL STEM CELL TRANSPLANTATION

One of the newer and exciting applications of AM has been its use as carrier for cultured limbal cells for ocular surface reconstruction procedures. It is now accepted that the surface epithelium regenerates

from progenitor stem cells located at the limbus (cornea) or fornices (conjunctiva), from which new cells migrate and differentiate into daughter cells.

An alternative therapeutic strategy that has been in vogue recently is the concept of expanding limbal epithelial cells *in vitro* using AM as a carrier or substrate. Schwab *et al* . earlier showed with similar technique of cultured limbal transplantation in 19 eyes (18 patients) 75% success with ocular surface destruction with no complications.

Expanding corneal epithelial cells from limbal biopsies *ex vivo* for use in ocular surface damage offers many significant advantages. One of them being only a small amount of limbal tissue is harvested from the uninvolved eye. Conventional limbal allografts require up to 12-clock hrs of limbal tissue and have the potential risk for limbal deficiency developing in the donor eye. Several studies reported excellent outcome of transplantation of cultivated limbal stem cell on denuded AM .

Amniotic membrane as a carrier in this instance has several distinct advantages over the other substrates that have been used. The basement membrane of AM contains Type IV collagen and laminin which plays an important role in cell adhesion. Further, it acts as a

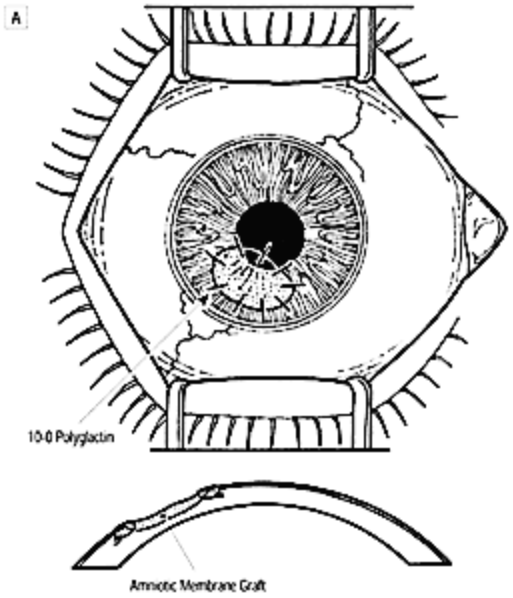


Fig.7 - Inlay technique

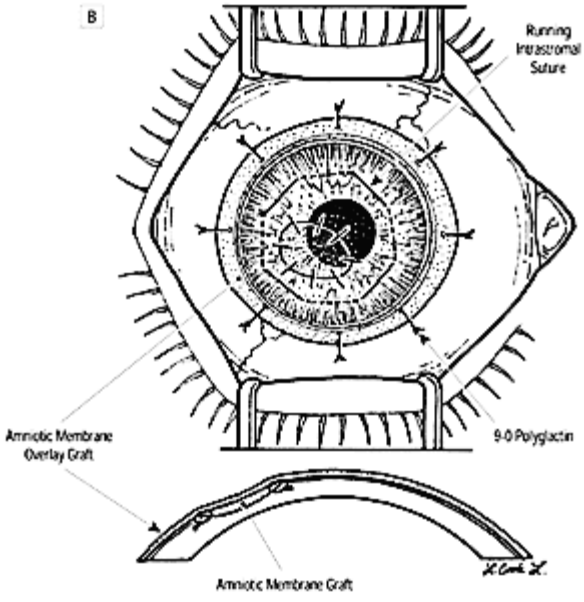


Fig.8 -overlay technique

natural substrate for the cell growth and when transplanted gets integrated onto the corneal surface. It also enables easier handling during transplantation. Tseng *et al* . proposed that culturing the explants on an intact AM with devitalized epithelium favors expansion of an epithelial phenotype that closely resembles limbal stem cells.[7,8]

CLINICAL APPLICATIONS:

FOR CONJUNCTIVAL DISEASE:

- Pterygium
- Bulbar conjunctival reconstruction after removal of large lesions or scars
- Conjunctivochalasis
- With or without preserved sclera or pericardium for bleb leakage or revision.
- scleral melt
- lid reconstruction
- orbit reconstruction

FOR CORNEAL DISEASE

- Persistant corneal epithelial defect with or without ulceration
- Partial limbal stem cell deficiency
- Total limbal stem cell deficiency
- For chemical burns,Steven-Johnsons syndrome
- Bullous keratopathy with erosion
- Band keratopathy

COMPLICATIONS

It has an excellent safety profile. Ocular infection is very rare but is the most common complication after non preserved amniotic membrane transplant. Post-operative complications following the use of preserved amniotic membrane is very rare. Hypopion formation in three cases have been reported. It was a non-infectious hypopion which represents a local immune reaction after sensitization of foreign antigens of the previous amniotic membrane from the same donor. Using amnion from a different person for a repeat graft will prevent this. Corneal calcification were reported in some. Bacterial keratitis with Staphylococcus aureus was reported in one case.

PART - 2

AIMS OF THE STUDY

a) To assess the immediate improvement in symptomatology of the patient.

b) To assess the effect of the amniotic membrane in the faster healing of ulcers.

c) To delay or to avoid the requirement for a therapeutic kertoplasty

INCLUSION CRITERIA

- Smear positive uncomplicated bacterial keratitis
- Smear positive uncomplicated fungal keratitis
- Mixed ulcers
- Mild and moderate ulcers

EXCLUSION CRITERIA

- Descemetocoele
- Perforated ulcers
- Viral keratitis,Acanthamoeba keratitis
- Patients less than eighteen years of age
- Women of child bearing age
- Diabetics

METHODS AND METHODOLOGY

The study was conducted at cornea services of the Regional Institute of Ophthalmology, Government Ophthalmic hospital, Chennai between May 2010 and November 2011.

21 eyes of 21 patients who underwent Amniotic membrane grafting were included in the study. These patients underwent Amniotic membrane grafting for Corneal Ulcers.

Patients who showed smear positive bacterial and fungal corneal ulcers were subjected to single layered amniotic membrane grafting depending on the severity of the ulcer. We did it for mild and moderate ulcers. We defined mild ulcers as less than 2 mms in size and involving the superficial stroma and moderate ulcers 2-5 mms in size and involving the mid-stroma. A thorough slit lamp examination and visual acuity testing with. Snellens chart was done pre and post-operatively.The patients were enquired about the severity of their symptoms,more with respect to the severity of their pain.

Smear was done for all patients presenting with corneal ulcers, both gram staining and KOH mount. They were treated with topical antibiotics and anti-fungals for one week and then single layered

amniotic membrane grafting was done. We used as control a group 21 almost similar mild and moderate smear positive ulcers with an equal distribution of bacterial, fungal and mixed as the study group from the year 2008 and 2009 from our old records and comparisons were made. The membrane was obtained from the placentas of patients undergoing elective lower segment caesarian section from the institute of obstetrics and gynaecology after screening for hepatitis B and HIV.

Under peribulbar block, the ulcer was first debrided. A suitable size of the amniotic membrane was cut such that it could cover the entire cornea and a few millimeter of the conjunctiva. The overlay technique was used. A few pricks were made in the membrane before placing it on the cornea using a 26G needle to prevent air locking behind the membrane and the membrane lifting up. The membrane was then placed on the cornea and then sutured to the conjunctiva with 10-0.A pad and bandage was applied. The eye was patched for 24 hours and then antibiotics were continued. The graft was found to be retained for an average of five days after which it sloughed. The conjunctival sutures were then removed History regarding the patients symptoms both pre and post operatively mainly pain, was taken into account to determine symptomatic improvement which was the primary aim of the study.

Redness and photophobia were also considered and the patient was examined under slit lamp regularly, to look for signs of healing. Visual aquity testing was also repeated after five days. The symptoms were graded as follows.

Grades of pain :

Grade 0 ; no pain ,

Grade 1; occasional mild pain,

Grade 2; constant mild pain,

Grade 3; moderate to severe pain,

Grade 4 ; constant severe pain.

Grades of redness :

Grade 0 ; no redness,

Grade 1; redness in one quadrant,

Grade 2; redness in two quadrants,

Grade 3; redness in four quadrants,

Grade 4; redness all around

Grades of photophobia :

Grade 0; no photophobia,

Grade 1 ; photophobia only in bright light,

Grade 2; photophobia in day light,

Grade 3; photophobia in dim light,

Grade 4; photophobia without light.

Ulcers complicated by descematocoele and perforated ulcers were excluded from the study. Viral keratitis was not included. Known diabetics and newly diagnosed diabetics with fasting blood sugar more than 130 milligrams were also excluded.

Women of child bearing age were not taken. Visual acuity with Snellen's chart, corneal smear, slit-lamp photography was done pre and post-procedure. The patient history regarding an improvement in symptoms was taken the next day and the patient was observed for signs of healing. The patients were followed up over a period of 3months.Most of them were lost from follow up.

OBSERVATION AND ANALYSIS

Table - 2 : IMPROVEMENT IN SYMPTOMS

			Group		Total
			Control	**Study**	
Symptom (Pain) - Post op	No pain	Count	0	2	2
		% within Symptom (Pain) - Post op	.0%	100.0%	100.0%
		% within Group	.0%	9.5%	4.8%
	Occasional mild pain	Count	5	10	15
		% within Symptom (Pain) - Post op	33.3%	66.7%	100.0%
		% within Group	23.8%	47.6%	35.7%
	Constant mild pain	Count	2	5	7
		% within Symptom (Pain) - Post op	28.6%	71.4%	100.0%
		% within Group	9.5%	23.8%	16.7%
	Moderate	Count	9	3	12
		% within Symptom (Pain) - Post op	75.0%	25.0%	100.0%
		% within Group	42.9%	14.3%	28.6%
	Severe	Count	5	1	6
		% within Symptom (Pain) - Post op	83.3%	16.7%	100.0%
		% within Group	23.8%	4.8%	14.3%
Total		**Count**	**21**	**21**	**42**
		% within Symptom (Pain) - Post op	**50.0%**	**50.0%**	**100.0%**
		% within Group	**100.0%**	**100.0%**	**100.0%**

In the study group 2 patients showed a complete absence of pain following the procedure.71.4% of the patients had only mild pain. There was a significant reduction in the patients who had severe pain. It reduced from 47.6% pre-operatively to 4.8%. post-operatively.

Table – 3 : RATE OF HEALING

	Group	N	Mean	Std. Deviation	Std. Error Mean
No. of Days of Healing	Control	6	19.00	3.162	1.291
	Study	13	9.08	2.431	.674

In the group with AMG, healed ulcers took an average of 9.08 days to do so, where as the group with no AMG took 19 days to heal. By chi square test it was statistically significant. $p<0.001$

Table – 4 : OUTCOME OF THE PATIENTS

			Group		Total
			Control	**Study**	
Outcome	healed	Count	6	13	19
		% within Outcome	31.6%	68.4%	100.0%
		% within Group	28.6%	61.9%	45.2%
	TKP	Count	15	8	23
		% within Outcome	65.2%	34.8%	100.0%
		% within Group	71.4%	38.1%	54.8%
Total		**Count**	**21**	**21**	**42**
		% within Outcome	**50.0%**	**50.0%**	**100.0%**
		% within Group	**100.0%**	**100.0%**	**100.0%**

The need for a therapeutic keratoplasty in the study group decreased. There was a 33% reduction in the need for a TKP. P <0.03 by chi square test, which was significant.

DISCUSSION

With a view to achieving rapid epithelialization and immediate symptomatic relief by covering the raw surface with exposed nerve endings directly by amniotic membrane, we ventured to take up the present study. We were led to the idea of trying this membrane in cases of corneal ulcers, more so because any material which does not behave as a foreign body over the eye should provide the most suitable coverage material. It has an added advantage that it can be easily prepared and stored in any hospital or laboratory.

Prior to inserting such a graft it is of utmost importance to combat the infection because this immediate covering material worsens the condition of an ulcer, if it is grossly infected or sloughing.

From the above data it was very remarkable to find such quick relief of pain. There was an immediate relief in symptoms in the first two hours, this relief of pain was due to the effect of anaesthesia but later on when the effect wore off 84% per cent of the patients showed a remarkable improvement in symptoms (Fig.10). This cause of immediate relief of pain in these cases must be due to the protective covering over the exposed nerve endings of the ulcerated corneal

surface. This immediate covering material probably acted by not allowing any irritant, mechanical, chemical or toxic to reach the nerve endings. The cases where pain was continued for a longer period may be due to an inflammation of the iris. In the control group only 33.33% of the patients had an improvement in symptoms after topical antibiotics alone.

In cases where amniotic membrane grafting was done there was marked reduction in the healing time. The average healing time came down to 9.08 days as against 19 days in control cases (Fig.13).This time was calculated from one week of application of topical medication after which grafting was carried out and antibiotics continued in the control group. A healing of the epithelial defect and absence of the infiltrate and circum-corneal injection with scarring were taken as indicators of healing. The various factors present in the membrane was probably the reason for this. Besides, as the ulcer is protected, the mitosis of corneal cells and production of fibroblasts to fill up the ulcer proceeded unimpaired. Moreover the mechanical trauma on the diseased cornea by blinking of the lids is also eliminated, thus allowing the process of healing to proceed unimpaired or even to hasten. Thus increased metabolic activity, and avoidance of irritants may be factors in

enhancing the healing process. In our series of clinical cases it was obvious that the healing process was definitely quickened by the grafting. Out of the 21 cases in the study group 9 were bacterial, 7 fungal and 5 mixed ulcers (Fig.9). 13 cases healed and 8 underwent a TKP.

Among the ones that healed there were 7 bacterial (77.77%), 4 fungal (57.14%) and 2 mixed (40%) (Fig.12). In these cases the graft probably reduced the chances of a perforation by maintaining the tectonicity of the globe for some time and delayed the emergent need for a TKP and thereby helped us, as the supply of donor eyes is still not meeting our requirements.Our study shows a better response with respect to bacterial ulcers. Of the control group, in 6 patients the ulcer healed and 15 patients underwent a TKP of which three perforated before we could undertake one.(Fig.11) Of the healed ulcers 3 were fungal, 2 bacterial and 1 mixed.

Visual outcome was not taken into account in either groups. Some of them in the study group even showed a decrease in vision due to the formation of thick opacities due to healing. In the control group also we took mild and moderate ulcers with an equal distribution of bacterial, fungal and mixed as the study group.

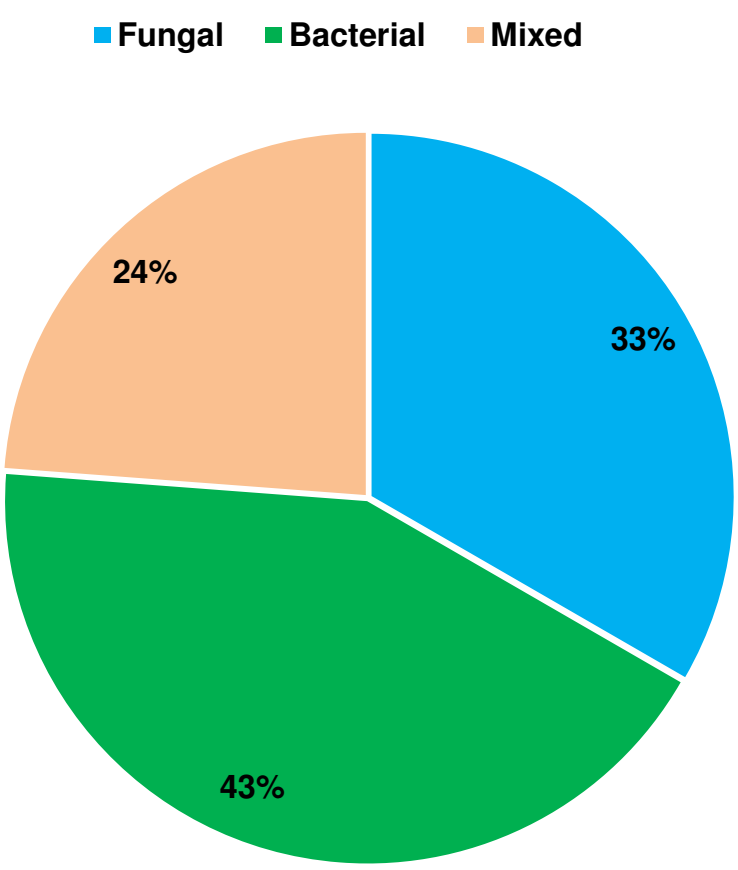


Fig.9- Etiological distribution of the ulcers

Table- 6 : SYMPTOMATIC IMPROVEMENT

	NO.	PERCENTAGE
AMG	13	84%
NO AMG	6	33%

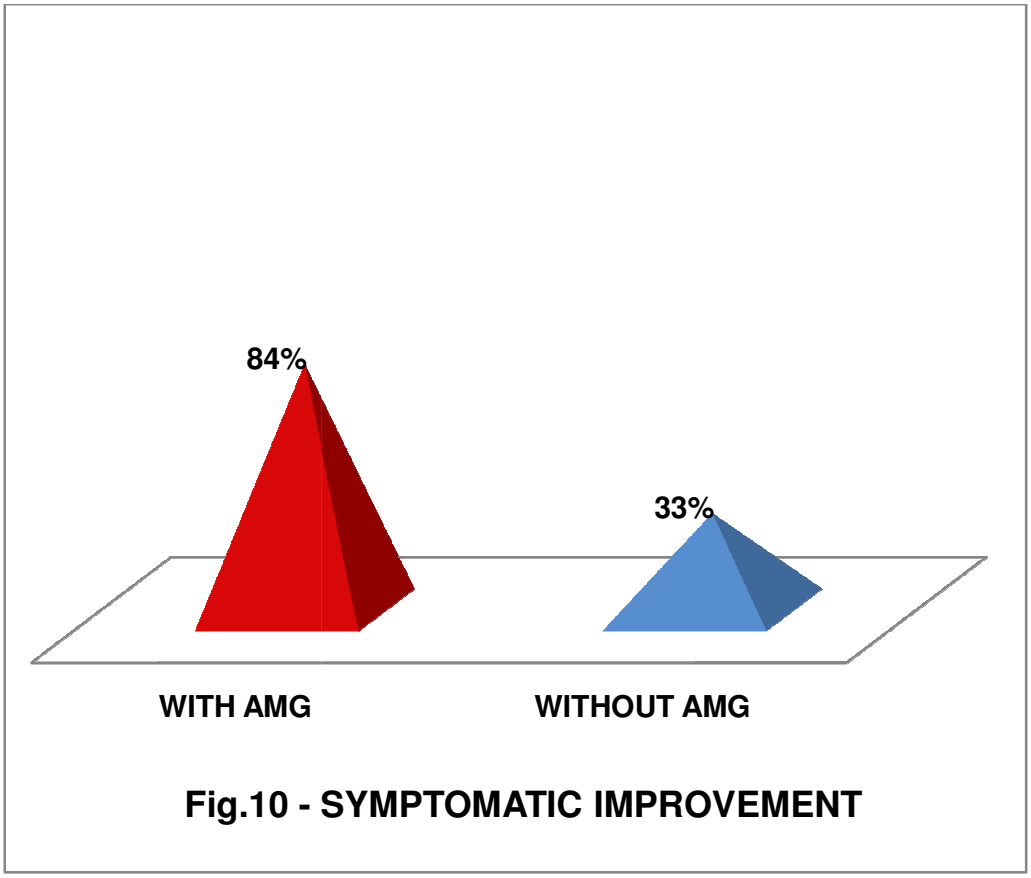
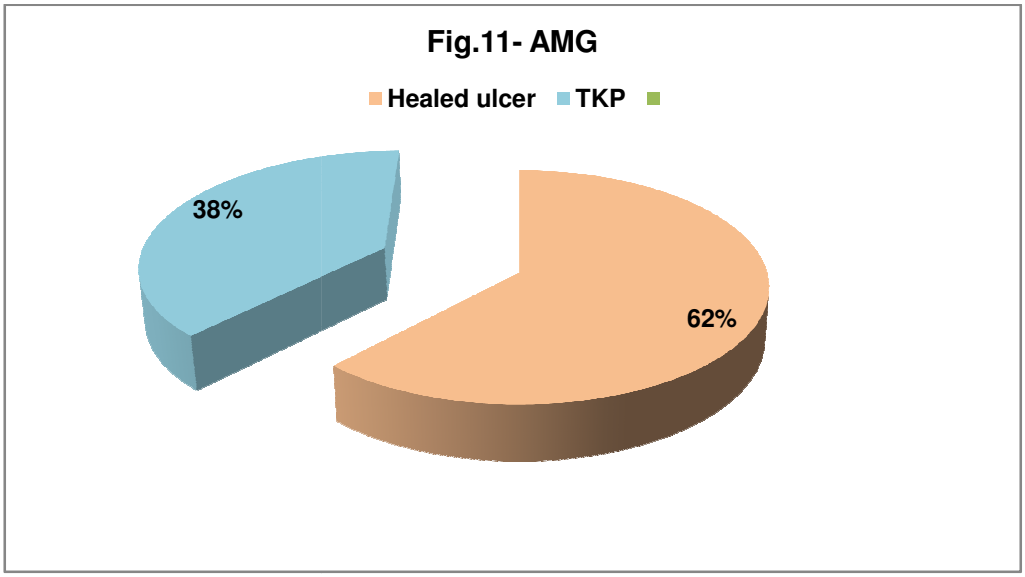


Fig.10 - SYMPTOMATIC IMPROVEMENT

TABLE - 7 : RESPONSE TO TREATMENT

	HEALED	TKP
AMG	62%	38%
NO AMG	29%	71%



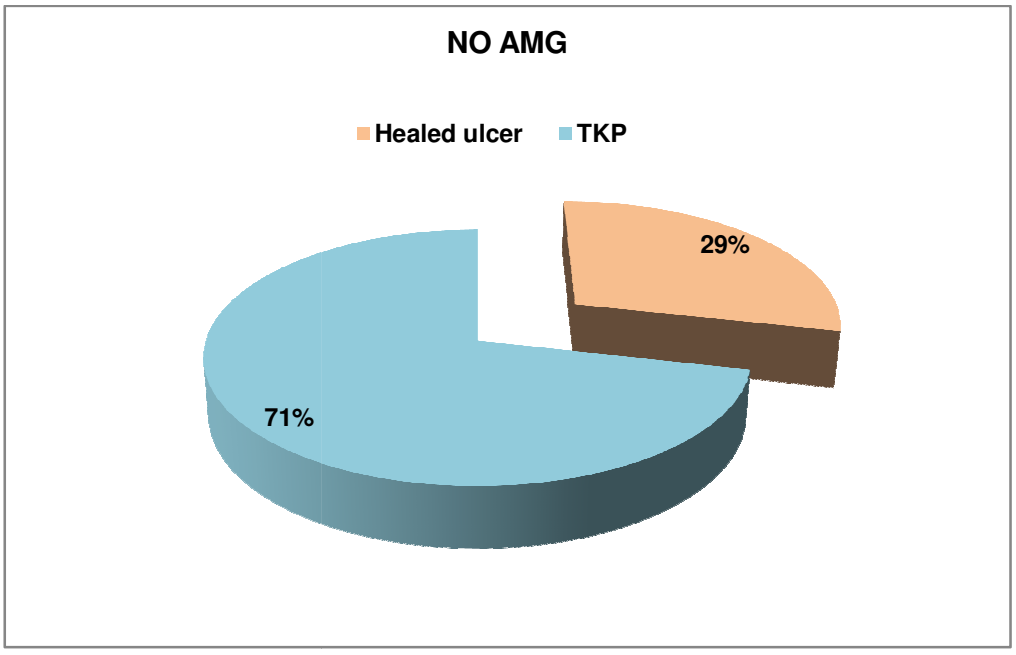
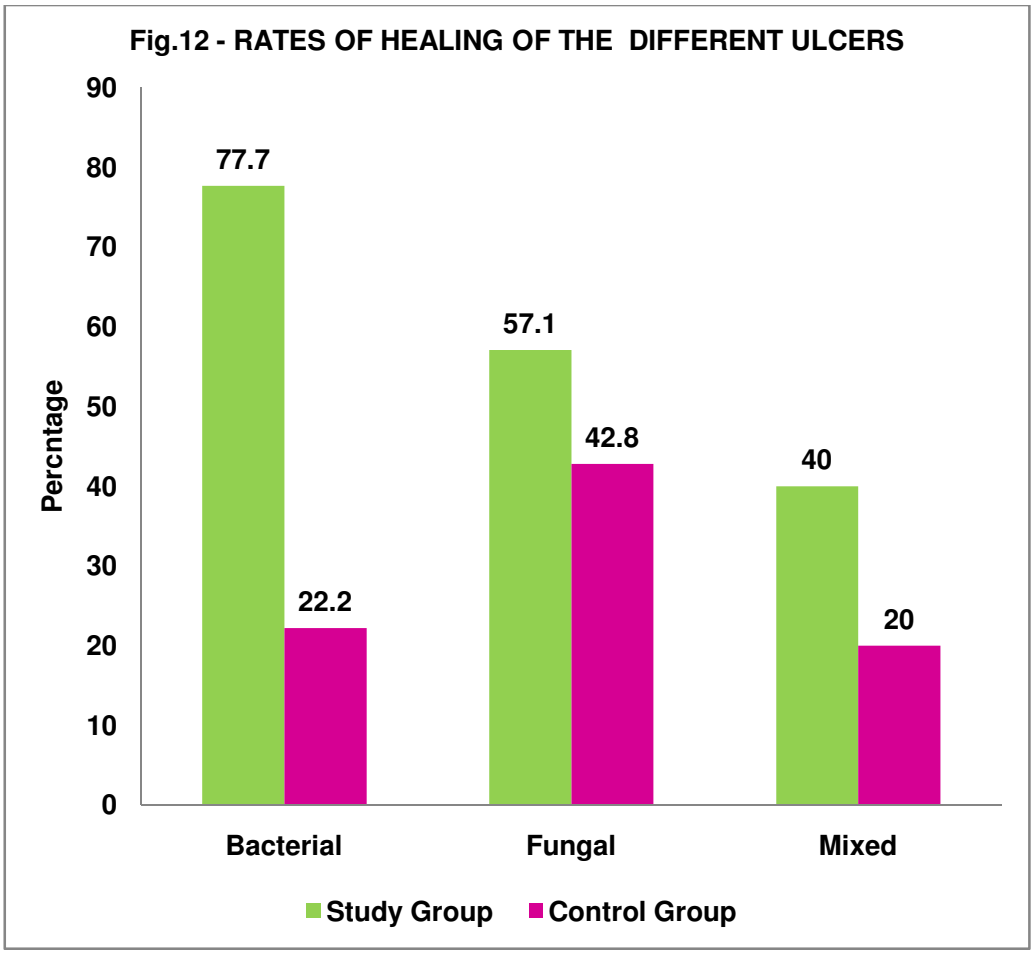


Table – 8: Rate of Healing of the Different Ulcers

Etiology	Study Group			Control Group		
	Total	Healed	%	Total	Healed	%
Bacterial	9	7	77.77%	9	2	22.22%
Fungal	7	4	57.14%	7	3	42.8%
Mixed	5	2	40%	5	1	20%



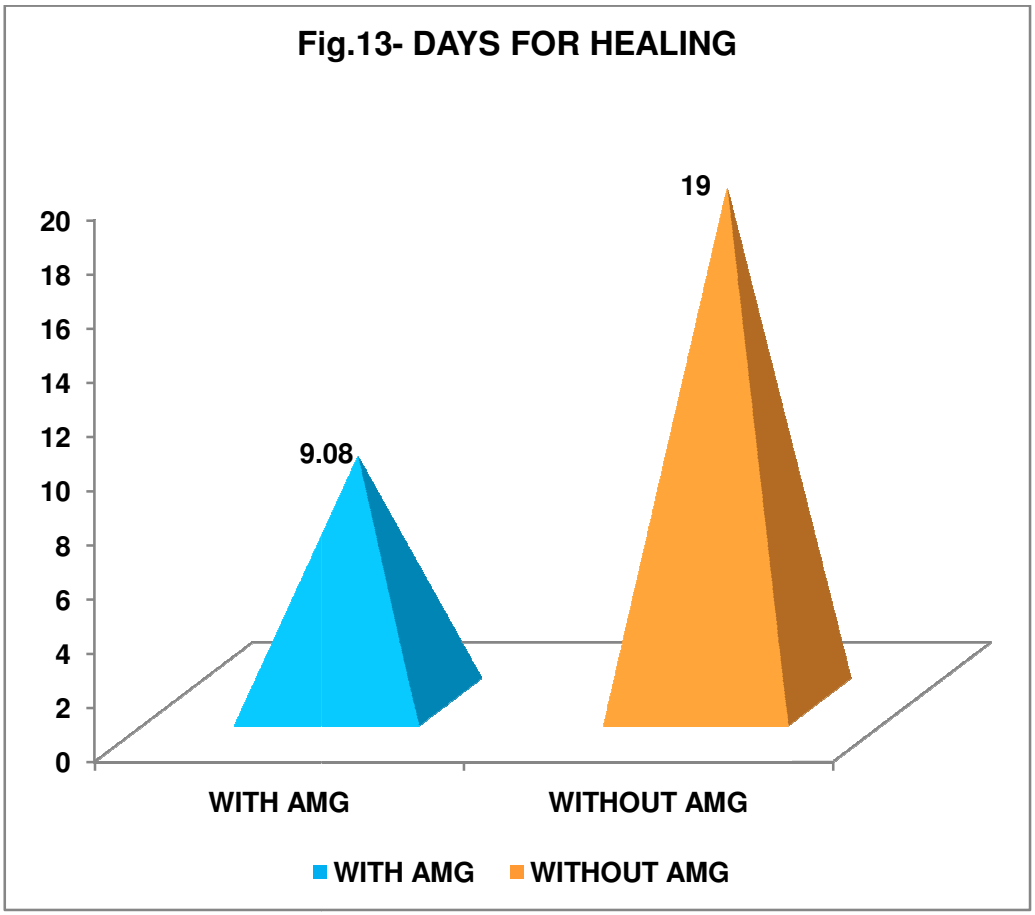


Fig.13- DAYS FOR HEALING
20
18
16
14
12
10
8
6
4
2
0
9.08
19
WITH AMG
WITHOUT AMG
WITH AMG
WITHOUT AMG

REVIEW OF LITERATURE

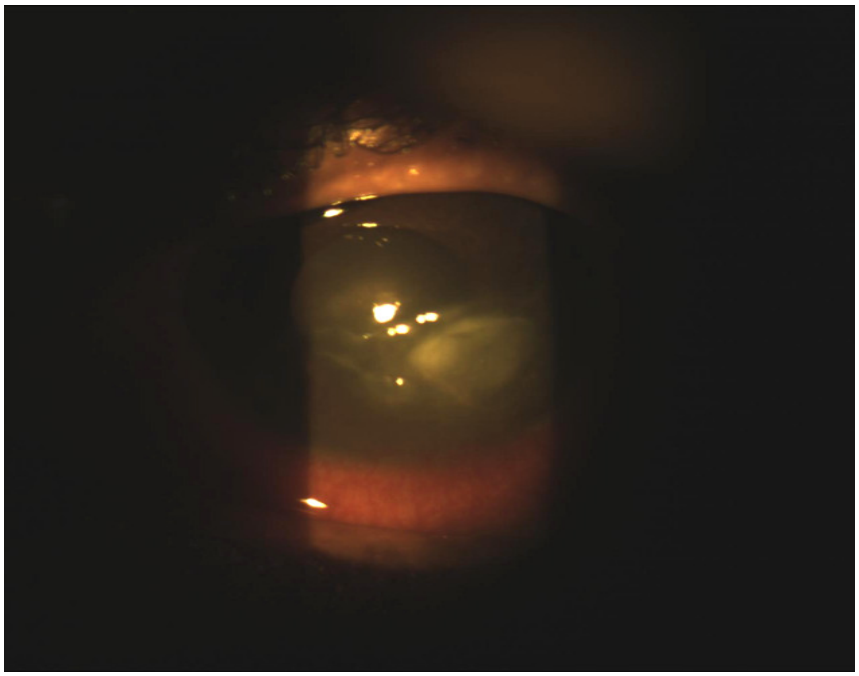
A similar study was performed by Jae-Soon Kim,et al in the year 2010. A prospective study of 21 consecutive eyes (21patients) was performed. Sufficient antibacterial, antifungal, or antiviral agents were applied to eradicate causative organisms before permanent or temporary amniotic membrane transplantation, or a combination of the two in few patients. The amniotic membrane was soaked in antiinfective agents before transplantation. The follow up ranged from 4 to 28 months (mean, 18 months). They included even viral and Acanthamoeba keratitis.The corneal surface was healed successfully and recurrences of microbial infection were not noted in any case. Visual acuity was improved in cases that were nonscarring or after additional penetrating keratoplasty.They concluded that Amniotic membrane transplantation seemed to be a useful adjunctive surgical procedure for the management of infectious corneal ulcer by promoting wound healing and reducing inflammation.

Shukla IMet al conducted a similar study in 72 patients, Indian J Ophthalmol 1962 but he applied it on both superficial and deep ulcers. In superficial ulcer group the average healing time came down to 6 days as against 11.8 days in control cases and similarly in deep ulcer cases

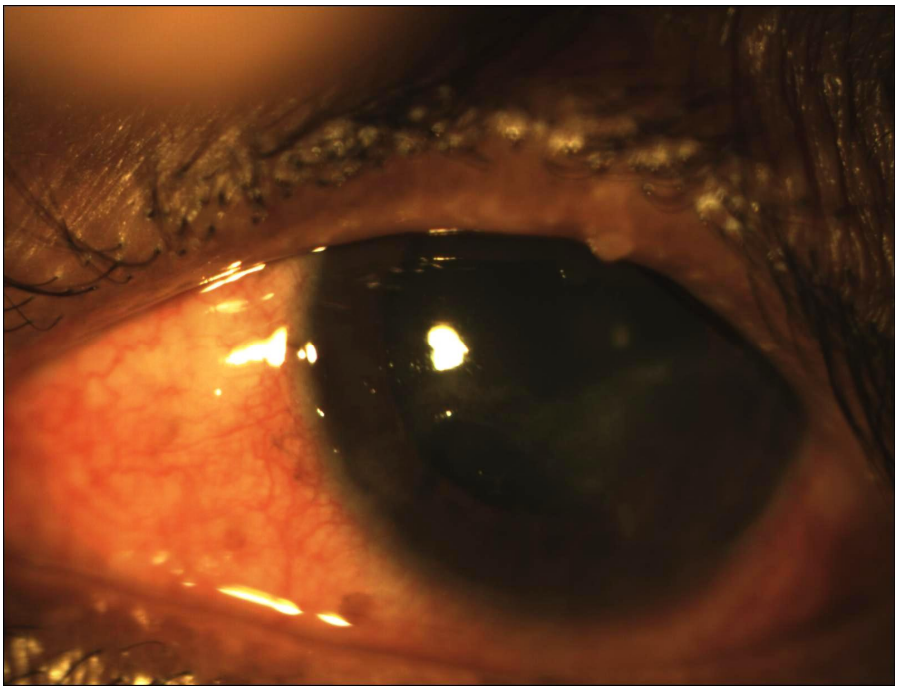
the average time came down from 19.5 days to 9.6 days where grafts were applied on.

In our study we have taken only superficial ulcers. In our study, the time for healing was 9.2 days as against 18.7 days in the control group. We have taken only ulcers of bacterial, fungal and mixed etiology.

Fig-14

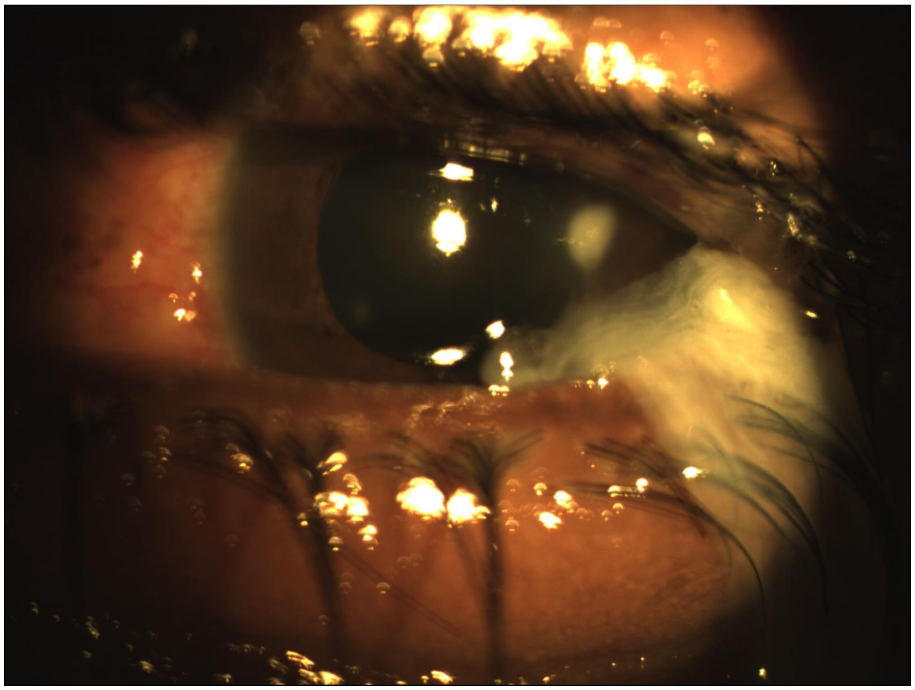


Pre AMG

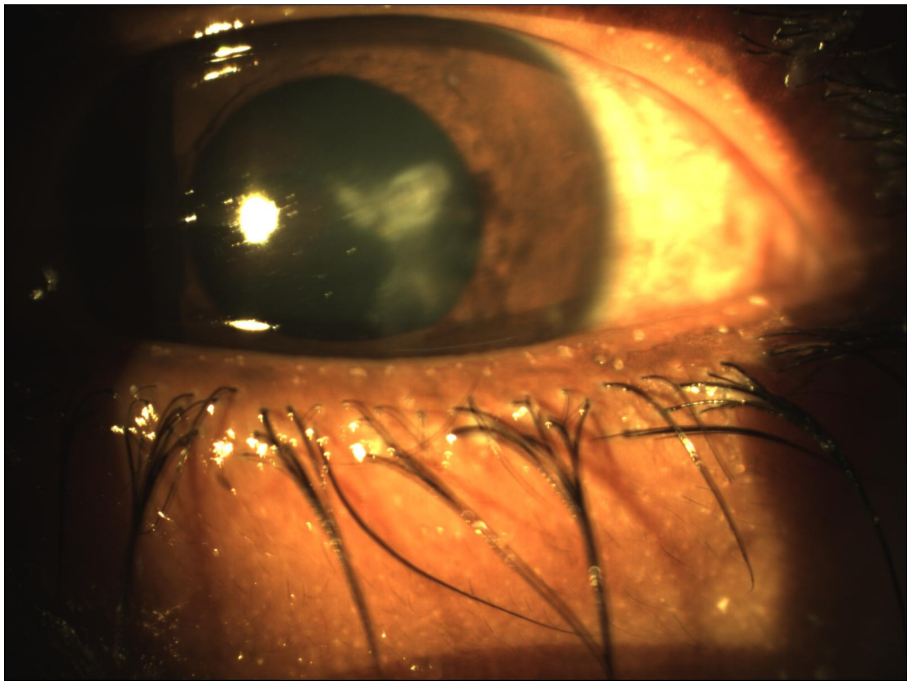


Post AMG (healed)

Fig-15

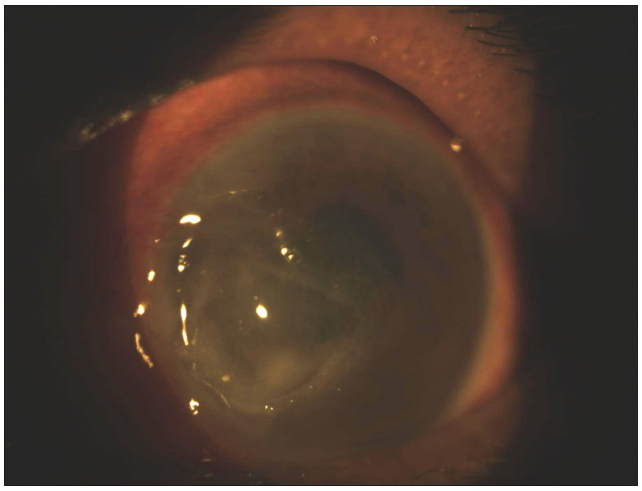


Pre AMG (The graft is seen sloughing out)

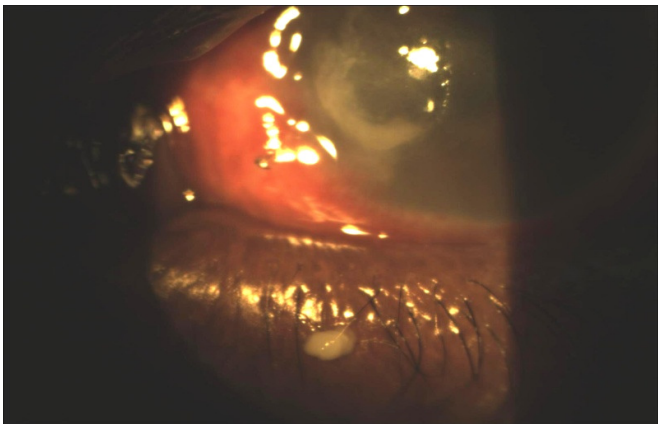


Post AMG (HEALED)

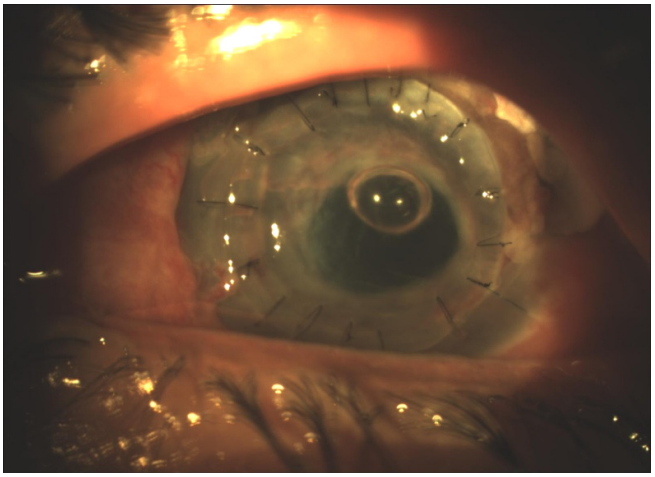
Fig-16



Pre AMG



Post AMG (NOT HEALED)



TKP

CONCLUSION

Our study proves that amniotic membrane grafting is indeed an effective treatment modality in ulcers especially in providing symptomatic relief. It reduces the time required for healing and thereby prevents the various complications associated with non healing ulcers. The need for a therapeutic keratoplasty is significantly low and thereby the various complications associated with it may also be avoided.. Though some of our patients showed really thick opacities probably an effect of the amniotic membrane itself, this is of no consequence as an optical keratoplasty can be undertaken later with respect to improving vision. The response seems to be better with respect to bacterial corneal ulcers.

As the amniotic membrane is easily available and there are no complications associated, it proves to be a useful measure for the treatment of corneal ulcers.

PART - 3

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PROFOMA

Name ;

Date :

Address ;

Age :

Sex :

Date of admission :

Date of discharge :

IP number :

Complaints

Pain-severity :

Present history :

Past history :

Family history :

History of diabetes mellitus and hypertension

General examination

LOCAL EXAMINATION (both eyes)

Visual axis

Uncorrected and Best corrected visual acuity

Lids and adnexa

Conjunctiva

Cornea-epithelial defect, depth of infiltrate

Anterior chamber-presence or absence of inflammation

Iris

Pupil

Fundus

Intraocular pressure

Corneal smear

Blood sugar

Blood sugar

Urine-albumin, sugar

Syringing of the Nasolacrimal duct

At each visit-Visual acuity

Slit lamp

MASTER CHART

S. No.	Name	AGE	SEX	DOA	DOS	SMEAR	Vn			SYMPTOMS (PAIN)	OUTCOME	DAY OF HEALING
							PRE-OP	POST-OP	PRE-OP	POST-OP		
	STUDY GROUP											
1)	Kanniammal	67	F	05-07-2010	14/5/10	F	4/60ph6/60	4/60nip	4	1	0	12
2)	Venkatesh	28	M	21/5/10	28/5/10	B	6/36nip	6/36nip	3	1	1	
3)	Veeraya	60	M	06-03-2010	06-10-2010	F	6/60ph6/36	6/60nip	3	1	0	9
4)	Palani	54	M	17/6/10	24/6/10	F	2/60nip	4/60nip	4	4	1	
5)	Mary	61	F	20/7/10	27/7/10	M	4/60ph6/60	6/60ph6/36	4	1	0	13
6)	Muniammal	63	F	21/8/10	28/8/10	B	5/60nip	4/60nip	4	1	0	12
7)	Ponniah	51	M	09-04-2010	09-11-2010	F	2/60nip	HM	3	3	1	
8)	Malakondaiya	68	M	10-03-2010	10-10-2010	B	3/60nip	2/60PH6/60	4	1	0	7
9)	Mari	41	M	10-08-2010	15/10/10	M	4/60nip	2/60NIP	3	1	1	
10)	Devan	65	M	03-03-2011	03-10-2011	M	1/60nip	1/60NIP	4	2	1	
11)	Mariammal	52	F	15/5/11	22/5/11	B	6/60ph6/36	6/36PH6/18	3	1	0	8
12)	Chengalvarayalu	45	M	21/5/11	28/5/11	M	6/60ph6/36	6/18PH6/12	3	0	0	5
13)	Veerasamy	67	M	06-04-2011	06-11-2011	B	6/36nip	6/24PH6/12	3	2	0	8
14)	Ponniah	72	M	06-11-2011	18/6/11	F	3/60nip	HM	3	4	1	
15)	Devi	41	F	15/7/11	22/7/11	F	CFCF	4/60NIP	4	2	0	10
16)	Udhayakumar	40	M	20/9/11	27/9/11	B	6/60ph6/36	6/60PH6/24	2	0	0	6
17)	Ponnan	69	M	09-08-2011	14/9/11	F	6/36nip	6/36PH6/12	4	2	0	8
18)	Velu	64	M	23/9/11	29/9/11	M	5/60nip	6/60ph6/36	4	3	1	
19)	Ravi	38	M	28/9/11	10-05-2011	B	4/60ph6/60	4/60NIP	4	1	0	9
20)	Parameshwari	35	F	28/9/11	10-05-2011	B	6/36ph6/24	6/12ph6/9	3	1	0	11
21)	Esther	73	F	11-06-2011	13/11/11	B	2/60nip	HM	3	3	1	

MASTER CHART

S. No.	Name	AGE	SEX	DOA	DOS	SMEAR	Vn			SYMPTOMS (PAIN)	OUTCOME	DAY OF HEALING
							PRE-OP	POST-OP	PRE-OP	POST-OP		
	CONTROL GROUP											
1)	Raman	47		05-01-2008		B	4/60NIP	6/36NIP	3	1	0	17
2)	Mary	51		06-07-2008		F	6/60PH6/36	6/60NIP	3	4	1	
3)	Muniammal	55		23/6/08		F	5/60NIP	5/60NIP	4	4	1	
4)	Kaniammal	67		07-03-2008		F	6/60NIP	6/60NIP	3	3	1	
5)	Rahman	41		07-05-2008		F	3/60PH4/60	3/60PH5/60	2	1	0	19
6)	Lakshmi	52		08-06-2008		M	6/60PH6/36	6/60NIP	3	3	1	
7)	Manikandan	61		31/8/08		F	6/36NIP	6/36NIP	3	4	1	
8)	Manickam	67		22/9/08		M	6/60NIP	6/60NIP	2	3	1	
9)	Veeralakshmi	56		27/9/08		B	2/60NIP	6/60PH6/36	2	1	0	15
10)	Suresh	59		10-07-2008		B	3/60PH6/60	3\60NIP	2	3	1	
11)	Seemati	60		17/10/08		M	6/60NIP	6/60PH6/36	2	3	1	
12)	Kruppan	63		21/11/08		B	6/36NIP	6/60NIP	3	3	1	
13)	Jeevan	51		12-05-2008		M	4/60PH6/60	6/36PH6/24	3	4	1	
14)	Ponnan	67		01-03-2009		M	4/60NIP	HM	4	1	0	21
15)	Kasthuri	49		02-05-2009		B	3/60PH6/60	6/60NIP	2	3	1	
16)	Kaliyaperumal	62		21/3/09		F	3/60NIP	2/60NIP	2	2	1	
17)	Dasran	55		16/4/09		F	4/60PH6/36	6/36PH6/24	4	3	0	24
18)	Maran	60		05-12-2009		B	4/60PH6/60	6/24PH6/18	4	2	0	18
19)	Lakshmi	71		19/5/09		B	6/36NIP	4/60PH6/60	3	1	1	
20)	Paramehwari	57		26/6/09		B	3/60NIP	CFCF	3	4	1	
21)	Muniammal	53		08-07-2009		B	2/60NIP	HM	2	3	1	

Key to master chart-

MASTER CHART

S. No.	Name	AGE	SEX	DOA	DOS	SMEAR	Vn			SYMPTOMS (PAIN)	OUTCOME	DAY OF HEALING
							PRE-OP	POST-OP	PRE-OP	POST-OP		

Smear,F-fungal,B-bacterial,M-mixed.OUTCOME-0-HEALED,1-Therapeutic keratoplasty,PAIN,0-no pain,1-occasional mild pain,2-constant mild pain,3-moderate pain,4-severe pain.

KEY TO MASTER CHART

SMEAR

F-fungal,

B-bacterial,

M-mixed

OUTCOME

-0-HEALED

1-Therapeutic keratoplasty

PAIN,

0-no pain,

1-occasional mild pain,

2-constant mild pain,

3-moderate pain,

4-severe pain

Vn-vision

CFCF-counting fingers close to face.

HM-Hand movements